Research Article

Effect of Simulated Microgravity Conditions on Differentiation of Adipose Derived Stem Cells towards Fibroblasts Using Connective Tissue Growth Factor

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Background: Mesenchymal stem cells (MSCs) are multipotent cells able to differentiating into a variety of mesenchymal tissues including osteoblasts, adipocytes and several other tissues.

Objectives: Differentiation of MSCs into fibroblast cells *in vitro* is an attractive strategy to achieve fibroblast cell and use them for purposes such as regeneration medicine. The goal of this study was investigate the simulated microgravity effect on differentiation of Adipose Derived Stem Cells (ADSCs) to fibroblasts.

Materials and Methods: To fibroblast differentiation 100 ng.mL^{-1} of connective tissue growth factor (CTGF), and for simulation microgravity, 2D clinostat was used. After isolation the human ADSCs from adipose, cells were passaged, and at passages 3 they were used for characterization and subsequent steps. After 7 days of CTGF and simulated microgravity treatment, proliferation, and differentiation were analyzed collectively by MTT assay, quantitative PCR analyses, and Immunocytochemistry staining.

Results: MTT assay revealed that CTGF stimulate the proliferation but simulated microgravity didn't have statistically significant effect on cell proliferation. In RNA level the expression of these genes are investigated: collagen type I (*COLI*), elastin (*ELA*), collagen type III (*ColIII*), Matrix Metalloproteinases I(*MMP1*), Fibronectin 1 (*FN1*), CD44, Fibroblast Specific protein (*FSP-1*), Integrin Subunit Beta 1 (*ITGB1*), Vimentin (*VIM*) and Fibrillin (*FBN*). We found that expression of *ELN*, FN1, *FSP1*, *COL1A1*, *ITGB1*, *MMP1* and *COL3A1* in both condition, and *VIM* and *FBN1* just in differentiation medium in normal gravity increased. In protein level the expression of COL III and ELN in simulated microgravity increased.

Conclusions: These findings collectively demonstrate that the simulated microgravity condition alters the marker fibroblast gene expression in fibroblast differentiation process.

Keywords: Adipose Derived Stem Cells (ADSCs), Differentiation, Extracellular Matrix (ECM), Fibroblast, Real Time PCR, Simulated Microgravity

1. Background

Fibroblasts are omnipresent cells that constitute the stroma of almost all tissues (1-3). In recent years, they are widely used for engineering of tissues such as skin and blood vessels due to their role in wound healing and secretion of extra cellular matrix (ECM) components (4-5). Because of ethical and safety problems in allogenic grafts and limitation in the amount of autogenic cells, nowadays stem cell differentiation has a much more attention for transplantation purpose (6).

Mesenchymal stem cells (MSCs) are multipotent cells with the ability to differentiating into a variety of mesenchymal tissues including osteoblasts, adipocytes, chondrocytes and several other tissues (7). They are adherent cells that proliferate in high rate and can be isolated from different adult tissues including adipose tissue (8). They are positive for CD105, CD90, and CD73 but negative for CD34, CD19, CD14, CD45, CD79a, and HLA-DR (7). Since adipose tissues are abundant and easy to access by less invasive methods,

they are ideal sources of adult stem cells.

Fibroblast cells are considered as mature MSCs (9). Remarkable variety and lack of unique molecular markers for fibroblasts caused some of the researchers conveniently consider uncharacterized cells as fibroblasts according to their morphology (10-12). An example of this case is bone marrow mesenchymal stem/stromal cells (MSCs) that initially considered as fibroblast cells (13, 14). Distinguish between fibroblast and mesenchymal cells are also very difficult. They have an extremely similar morphological appearance, they have many identical cell surface markers and they are both proliferated in considerable rate (15).

MSCs don't have a unique surface antigen that certainly differentiates these cells from fibroblasts. However, Stro-1 and CD146 have been introduced as specific markers for MSCs but these markers seem limited to MSCs derived from bone marrow or renal tissue, while MSCs derived from adipose, for example, didn't express these markers (16).

At the moment, the best way to discriminate MSCs from fibroblasts is based on functional characters of MSCs that is not seen in fibroblasts including self-renewal and multipotent differentiation capacity of MSCs. Studies have shown that the fibroblasts ability in these cases are much less than MSCs (16). Also another difference between MSCs and fibroblasts is fibroblast function in extra cellular matrix (ECM) formation (17). The high expression of ECM components like collagen type 1 (*ColI*), collagen type 3 (*ColIII*), elastin (*ELN*), fibronectin (*FN*) and fibrillin (*FBN*) are known as one of the fibroblast markers (18) that can be influenced by mechanical stress (19).

Lee *et al.* (18) observed that in the presence of connective tissue growth factor (CTGF), MSCs may differentiate into cells expressing characteristics of fibroblast. They showed that differentiated cells lost their surface mesenchymal epitopes and their tri-lineage differentiation potential reduced strongly. Also the expression of fibroblastic markers such as Col-I, Col-III, FN and FBN increased at gene and protein level. CTGF, also known as CCN2, is a 36-38 kDa protein from CCN family of extracellular matrix-associated heparin-binding proteins (20). Studies have shown that CTGF affects fibroblast and mesenchymal cell ECM synthesis, proliferation, and adhesion (21).

2. Objectives

With regards to fibroblast function in ECM formation and the impact of mechanical stress on it, it is expected that changes in mechanical forces have a significant effect on the differentiation of MSCs to fibroblast. One of the most important mechanical factors that affect all type of life on earth is gravity (22). Microgravity has been confirmed to affect growth and physiology of cell through impacting on intracellular signaling mechanisms, cell secretions and gene expression (23-25). It has been shown that in microgravity cells tend to grow in 3D (dimentional) form, and it could help to differentiation process by mimic in vivo condition (22). Altogether, the aim of present work was to investigate the effect of simulated microgravity condition on fibroblast differentiation of adipose derived stem cells (ADSCs). We have examined the expression of fibroblastic marker genes in RNA and protein level. Our selected genes were *ITGB1* (integrin subunit beta 1), *ColIII*, *FBN1*, *VIM* (vimentin), *MMP1* (matrix metalloproteinase-1), *CD44*, *ColI*, *ELN, FSP1* (*fibroblast specific protein 1*). 2-D (2 dimensional) clinostat was used to simulate weightlessness.

3. Materials and Methods

3.1. Preparation of Adipose Tissue Sample and Isolation of Human ADSCs

All experiments were performed according to the Institutional Ethics Committee (IEC-SCTIMST). Tissue samples were acquired from 5 healthy women in the age group 34-48 years. Adipose samples were obtained from patients going through cosmetic liposuction and transferred to the Aerospace Research Institute in a sterile condition. After removing the blood phase, rest of the samples were washed with Hank's Balanced Salt Solution (HBSS; Biowest, France) and used for the cell isolation.

Cell isolation was done by enzymatic digestion method as described by Zhu *et al.* (8). Briefly, each sample was washed several times with HBSS for remove the blood residual. After washing fat specimens were incubated at 37 ºC for 60 min in HBSS (Biowest, France) containing 1 mg.mL^{-1} of collagenase type I (Sigma, USA) and shacked every 15 minutes. After enzymatic digestion, specimens diluted with an equal volume of serum (Biowest, France) -containing medium (Dulbecco's modified eagle medium) (DMEM; Biowest, France), cells were washed by centrifugation at 400 g for 10 min and suspension and floated lipid layer was discarded. The pellets, known as stromal vascular fraction (SVF), was washed and resuspended in DMEM medium supplemented with 10% fetal bovine serum (FBS), antibiotic-antimycotic (Biowest, France) solution and seeded into a 25 cm^2 cell-culture flask (TPP, Switzerland) and incubated at 37 °C in a

humidified atmosphere containing 5% CO_2 incubator. The following day, the medium was changed so the non-adherent cells and cellular debris were discarded and the adherent cells were preserved to achieve ~80% of confluence. The cells were passaged by a standard trypsinization (Biowest, France) protocol and cells at passage 3 were used for the analysis of ADSCs surface markers and differentiation experiments.

3.2. Characterization of Human ADSCs
Cultures of ADSCs were characterized by flowcytometry (FC). After trypsinization, Cells from passage 3 were suspended in phosphate-buffered saline (PBS) ($3 \times 10^5.100$ μL^{-1} for each reaction) and then incubated for 30 min at 4 ºC with the PE (phycoerythrin) and FITC-conjugated (fluorescein isothiocyanate) antibodies (BD Biosciences PharMingen, USA) against CD90-PE, CD105-FITC, CD73-PE as positive marker and CD34-PE ,CD45-FITC as negative marker. ADSCs were incubated for 20 min at room temperature in dark and then cells were washed with FC buffer to remove not-conjugated antibodies. Flowcytometric analyses were carrying out using a Cyflow Space (Partec) flowcytometer. Data were then analyzed by the FloMax software (version 2.70).

Functional characterizations of ADSCs were done by differentiation to adipocyte and osteoblast using Human Mesenchymal Stem Cell Functional Identification Kit (R&D systems, USA) according to the manufacturer's recommendations. To adipocyte differentiation, ADSCs were seeded in 6-well culture plate at an initial density of 2.1×10^4 cells per cm^2 containing α-MEM (Biowest, France) supplemented with antibiotic and 10% FBS to achieved 90-100% confluence. Then the medium was changed into differentiation medium comprise of α-MEM supplemented with adipogenic supplement (R&D systems, USA). Differentiation medium were replaced every 3 days. After 9 days, lipid vacuoles were observed and confirmed by Oil Red O staining of cytoplasmic lipid droplets.

To osteoblast differentiation ADSCs were seeded in 6-well culture plate at an initial density of 2.1×10^4 cells per cm^2 containing α-MEM (Biowest, France) supplemented with antibiotic and 10% FBS to achieved 50-70% confluence. Then the medium was changed into differentiation medium comprise of α-MEM supplemented with osteogenic supplement (R&Dsystems, USA). Differentiation medium were changed every 3 days. After 15 days, when cells start to detach, the osteocytes were prepared and it was confirmed by Alizarin Red S staining of extracellular matrix calcium deposition.

3.3. Differentiation to Fibroblast by CTGF Treatment
Passage 3 or 4 of ADSCs was seeded (about 2×10^4 cells per cm^2) in 96-well plates or tissue culture tube (TPP, Switzerland). At 80%-90% confluence, ADSCs were treated with 100 ng.mL^{-1} recombinant human CTGF (BioVendor, USA) (17) and 50 μg.mL^{-1} ascorbic acid (Sigma-Aldrich), and medium was change every third day.

3.4. Microgravity Simulation
To simulate microgravity, 2D clinostat was used. This device by rotating cells prevents them to feeling gravity. Rotation causes that gravity vector is not recognizable to cells. In the present study, ADSCs were seeded at a density of 2×10^6 cells on 25 cm^2 cell-culture flask or tissue culture tube (TPP, Switzerland) and at a density of 5×10^4 cells on 96-well plates. After cell adhesion, flasks filled completely by medium supplemented with antibiotic and 10% FBS to prevent the presence of air bubbles and 100 ng.mL^{-1} recombinant human CTGF for differentiated samples. To maintain the pH balance, the medium was supplemented with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The clinostat rotation speed was 20 rpm and the rotation times were 1, 3 and 7 days.

3.5. MTT Assay
The ADSCs were seeded in 96-well plates and were grown in microgravity condition for 7 days and the control group was grown in normal conditions (1g) for 7 days too. For differentiated group, MTT assay was done like the previous groups but after adding 100 mg.mL^{-1} recombinant human CTGF. A working solution of 5 mg.mL^{-1} (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT), (Atocel, Austria) dissolved in PBS was added to the culture medium (100 μl/well) to detect cell viability. After 3 h incubation, purple crystals of formazan were observed. The medium was removed and to solve formazan, 100 μL Dimethyl sulfoxide (DMSO) (Atocel, Austria) was added to each well. The amount of formazan produced from MTT cleavage was quantitated with an ELISA plate reader (BioTech Company, USA), at 570 nm wavelength.

3.6. Real-Time Quantitative PCR
On the seventh day, RNA was isolated from control group that differentiated in normal condition and microgravity group that differentiated under simulated microgravity condition. RNA extraction and cDNA synthesis were performed by CellAmp Direct RNA Prep Kit for RT-PCR (Takara, Japan) and PrimeScript RT reagent Kit (Takara, Japan) following

the manufacturer's procedure. To measure the gene expression, real-time PCR was performed using StepOnePlus Real-Time PCR (Applied Biosystems, USA) using SYBR green PCR master mix. Real-time PCR was programmed into three steps: initialization at 95 ºC for 2 min, denaturation at 95 ºC for 5 s, and annealing at 60 ºC for 30 s (total 40 cycles). For each gene to ensure specificity of PCR products, PCR melt curves were performed after real-time PCR constructed by sequential heating of the product. Primer sequences for collagen type I (*ColI*), elastin (*ELN*), collagen type III (*ColIII*), matrix metalloproteinases I (*MMP1*), fibronectin 1 (*FN1*), *CD44*, fibroblast specific protein 1 (*FSP1*), integrin subunit beta 1 (ITGB1), vimentin (*VIM*) and fibrillin (*FBN*) are listed in Table 1. All primers were synthesized by Macrogen (South Korea). Change in the fold number was calculated by using $2^{-\Delta\Delta Ct}$ method that was normalized by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the house keeping gene.

3.7. Fluorescence Immunocytochemistry

After 7 days of culture in simulated microgravity condition and CTGF treatment, collagen type 3 and elastin of the differentiated fibroblast cells was observed. Briefly, cultured cells on coverslips were fixed with 4% paraformaldehyde for 10 min and then washed three times with ice-cold PBS, followed by exposure to 0.01% Triton X-100 at room temperature for 5 min and then cells were washed in PBS three times.

ADSCs after clinorotation and CTGF treatment for 7 days were incubated with COLIII and ELN primary antibody (dilution 1:100; biorbyt) overnight at 4 °C. Then, primary antibodies were washed and fluorescent secondary antibody anti-Rabbit IgG Alexa Fluor (dilution 5 µg.mL^{-1}; Invitrogen) was added respectively for 1 h at room temperature in the dark. Subsequently, the cells were treated with Hoechst 33342 (dilution 1:100; Sigma) for 10 min. Images were obtained using fluorescent microscope (Olympus).

3.8. Statistical Analysis

To avoid errors due to differences in sample source, for each assay and analysis, cells from the same donor were used. Statistical correlation was performed using independent samples t-test and *p*<0.05 was considered statistically significant.

Table 1. Sequence of PCR primers.

Gene	Primer Sequence (5´ to 3´)	GeneBank No.	Amplicon size (bp)
Fsp1	Forward: CAGCTTCTTGGGGAAAAGGACA Reverse: CGATGCAGGACAGGAAGACAC	NM_002961.2	119
ELN	Forward: TTGTCTCCCATTTTCCCAGGTG Reverse: GTTGATGAGGTCGTGAGTCAGG	NM_000501.3	98
FN1	Forward: CTCGAAGAGCAAGAGGCAGG Reverse: CAACCGGGCTTGCTTTGAC	NM_212482.2	78
Col1	Forward: AATGGAGATGATGGGGAAGCTG Reverse: TGTTCCGGGCAATCCTCG	NM_000088.3	90
GAPDG	Forward: AACAGCCTCAAGATCATCAGCAA Reverse: GATGGCATGGACTGTGGTCAT	NM_001289746.1	120
ITGB1	Forward: TGCACCAGCCCATTTAGCTAC Reverse: CCTCCAGCCAATCAGTGATCC	NM_033668.2	168
CD44	Forward: GCTTCAATGCTTCAGCTCCAC Reverse: GGGTTGCTGGGGTAGATGTC	NM_000610.3	166
MMP1	Forward: TCACACCTCTGACATTCACCAAG Reverse: TCCCGATGATCTCCCCTGAC	NM_002421.3	79
VIM	Forward: TGAAAGTGTGGCTGCCAAGA Reverse: CAGAGAGGTCAGCAAACTTGGA	NM_001278918.1	71
ColIII	Forward: GGATGGTTGCACGAAACACAC Reverse: CAGGACCACCAATGTCATAGGG	NM_000090.3	116
FBN1	Forward: GAGTGCCTTGACAATCGGGA Reverse: GATTTGGTGACGGGGTTCCT	NM_000138.4	95

4. Results

4.1. Isolation and Culture of ADSCs

ADSCs were isolated from fat specimens obtained from patients during the course of abdominal surgery. 48 h after culture of SVF in cell culture flask, fibroblast-like spindle shape cells were observed (Fig. 1). Adhered cells proliferated with a considerable rate and get 70-80% of cell confluency after 4 days. The shape and adherence of cells conformed to mesenchymal stem cells characteristic.

4.2. Characterization of Human ADSCs

Flowcytometry analysis of the cells at passage 3 for mesenchymal stem cells marker showed that, ADSCs express CD90 (95.79%), CD73 (98.04%) and CD105 (96.35%) but not express CD34 (3.95%) and CD45 (3.72%) markers (Fig. 2). These results were showed that immunophenotypic markers of isolated cells are same with MSCs.
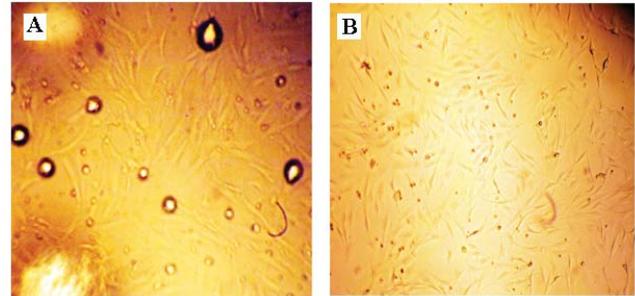
To demonstrate pluripotent capacity of ADSCs, they

Figure 1. Isolated ADSCs with enzymatic method from lipoaspirate. Fibroblast-like spindle shape cells were observed after 48 h culture of stromal vascular pellet (A). Oil spots are residue of digested fat (A). After changing medium, ADSCs in 40-50% of confluence were observed (B).

were differentiated to adipocyte and osteocyte. Staining results by Oil Red O to detect lipid-rich vacuoles and Alizarin Red S to detect calcium deposit production in extracellular matrix confirmed that the isolated cells could differentiate into adipocyte and osteoblast lineages (Fig. 2).
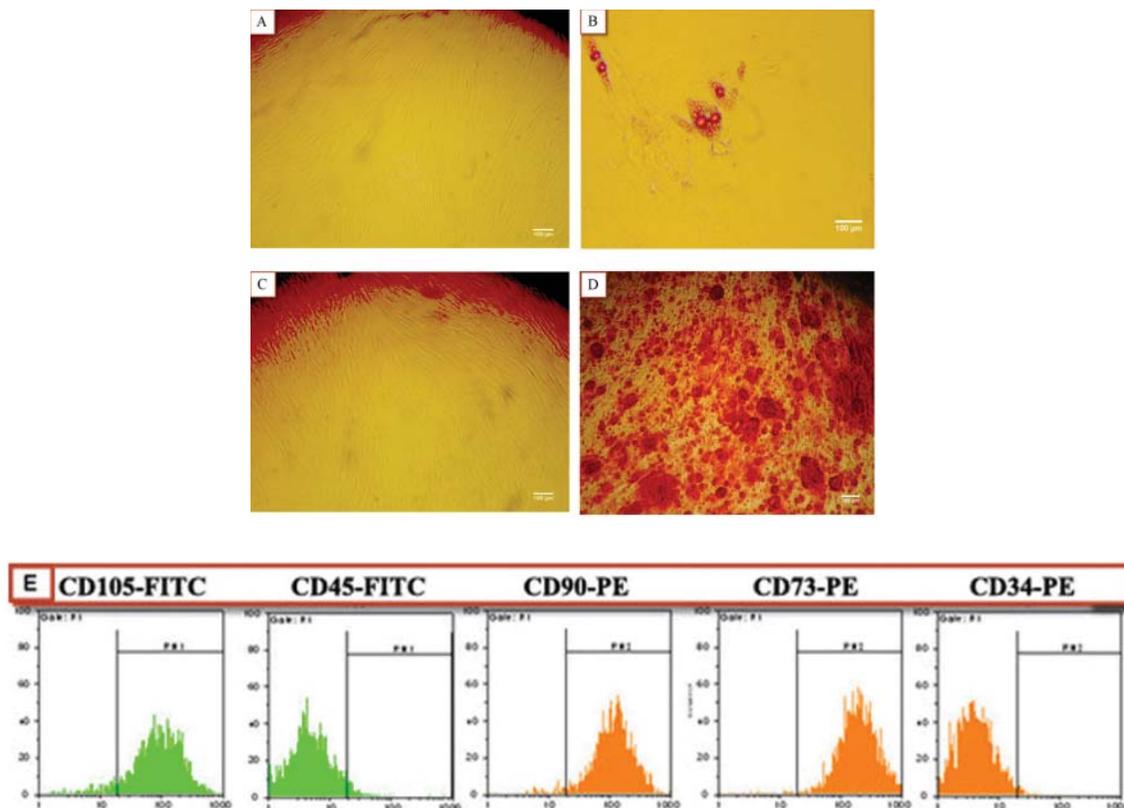

Figure 2. Characterization of ADSCs. Differentiation of human ADSCs to adipocyte and osteoblast lineages. (A and C) ADSCs cultured in control media; (B) ADSCs cultured in adipogenic media for 9 days and stained with Oil Red O; (D) ADSCs cultured in osteogenic media for 15 days and stained with Alizarin Red. (E) Immunophenotypic characteristics of human MSCs which were carried out using flow cytometry. Third-passage of isolated ADSCs is positive for CD90 (95.79%), CD73 (98.04%) and CD105 (96.35%) and negative for CD34 (3.95%) and CD45 (3.72%) markers.
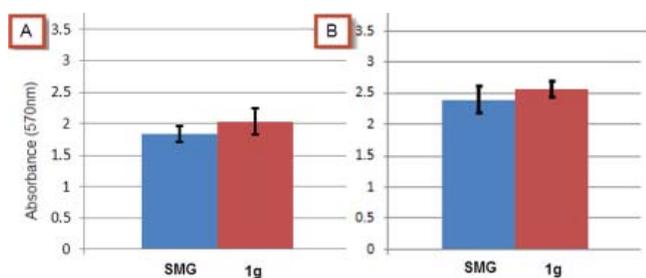
Figure 3. Cell viability of ADSCs before (A) and after (B) fibroblastic differentiation after 7 days in two conditions: simulated microgravity (SMG) and normal gravity (1G). There were no statistically significant changes in viability between the two culture conditions. p< 0.05 considered as significant.

4.3. Cell Viability Analyses by MTT Assay

The cell viability and the metabolic activity of the cells grown in the simulated microgravity condition in comparison to cells grown in normal condition and ADSCs that differentiated to fibroblast in the simulated microgravity condition in comparison to ADSCs that differentiated in normal condition were appraised using MTT assay. As seen in Figure 3 after 7 days in simulated microgravity condition cell viability didn't show significant differences between two culture conditions.

4.4. Real-Time PCR Analysis

We employed qRT-PCR to measure any changes in gene expression of fibroblast markers and ECM genes.

Our selected genes were: *GAPDH, ColI, Col III, FN1, MMP1, ELN, FBN1, FSP1, CD44, ITGB1* and *VIM* (Fig. 4).

As seen in Figure 4, the expression of *ELN, FN1, FSP1, Col1, ITGB1, MMP1* and *ColIII* significantly increased ($p \leq 0.05$) after differentiation of ADSCs to fibroblast in both normal condition and simulated microgravity condition (78%, 159%, 186%, 170%, 220%, 371%, 303% in normal condition and 98%, 140%, 243%, 245%, 543%, 512% and 587% in simulated microgravity, respectively). Our results showed that the expression of *VIM* and *FBN1* increased ($p \leq 0.05$) after differentiation of ADSCs (110% and 189%, respectively) but we could not detect any changes in their expression following microgravity treatment in comparison to normal condition ($p \geq 0.05$). In contrast to above genes, the expression of *CD44* decreased after differentiation of ADSCs to fibroblast in normal condition (39%). Our results indicated that following the microgravity treatment, the expression of *CD44* increased gradually (95%).

The greatest increase in expression was observed in *ColIII1* after CTGF treatment and 7 days exposure to simulated microgravity. The expression of *ColIII* increased up to 3 and 6 fold of control group in normal condition and simulated microgravity environment, respectively.

Expression of *FBN1* was increased in simulated microgravity but this increase was not statistically significant.
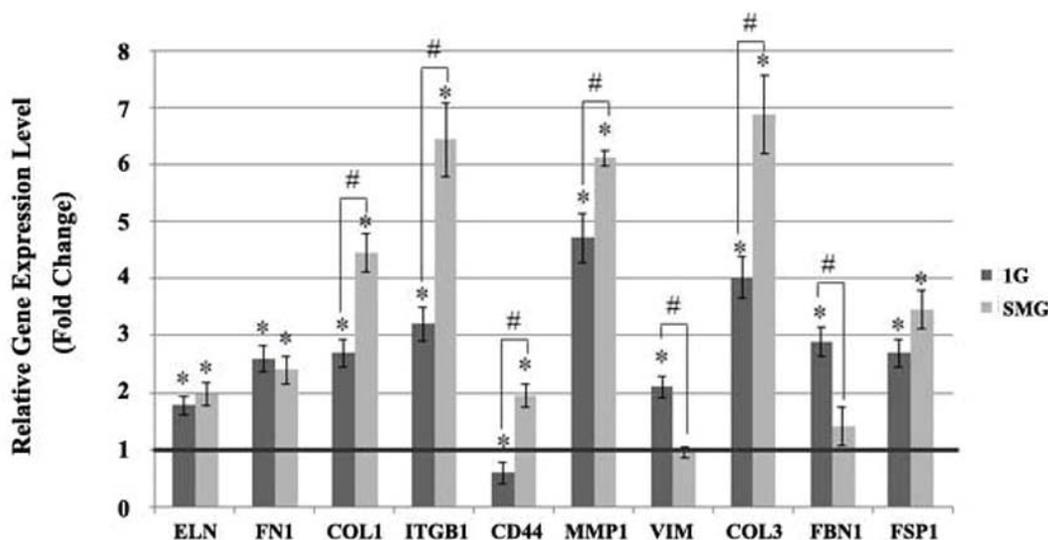

Figure 4. Effects of CTGF on gene expression of ADSCs cultured on simulated microgravity condition (SMG) and in normal condition (1G). Real-time quantitative PCR analysis was done after 7 days of culture. GAPDH was used as an internal control, and the gene expression was normalized to the CTGF-free culture that growth in normal gravity. * indicates significant difference ($p < 0.05$) relative to the CTGF-free culture (before differentiation to fibroblast). # indicates significant difference ($p < 0.05$) between differentiated ADSCs in 1G and MG condition.
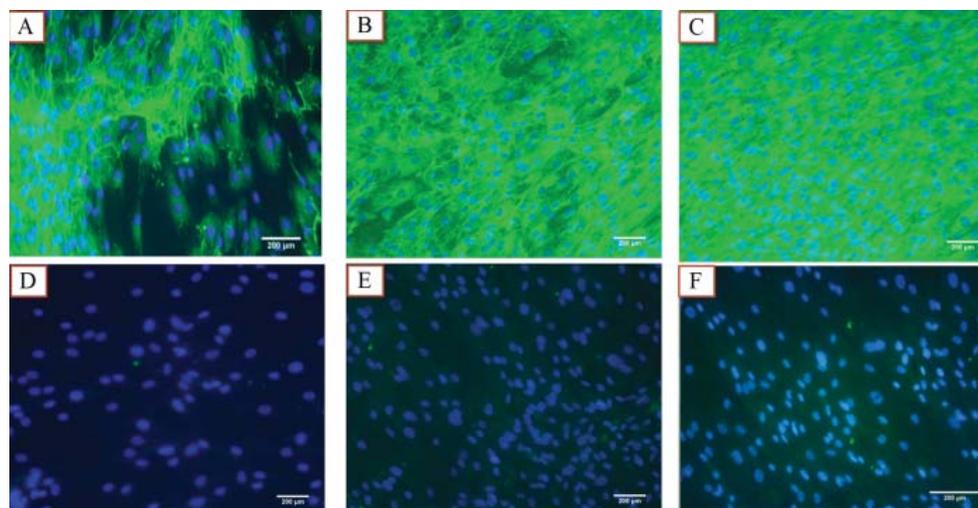
Figure 5. Immunocytochemistry analysis of collagen type III (A, B and C) and ELN (D, E and F) protein. (A and D) Immunofluorescence micrographs of samples before CTGF treatment. (B and E) Immunofluorescence micrographs of samples after CTGF treatment in normal gravity. (C and F) Immunofluorescence micrographs of samples after CTGF treatment in simulated microgravity environment.

4.5. Immunofluorescence Staining

ADSCs treated by CTGF and simulated microgravity were analyzed semi-quantitatively at the protein level using standard immunocytochemistry assays for collagen type III and elastin. As seen in Figure 5, it seems expression of ColIII protein after treated by CTGF in normal gravity (Fig. 5B) and simulated microgravity (Fig. 5C) condition was higher than the expression of it in normal condition (Fig. 5A). For elastin, this upregulation was seen just after simultaneously treatment by CTGF and simulated microgravity condition (Fig. 5F) but we could not detect any changes in its expression before (Fig. 5D) and after (Fig. 5E) treatment by CTGF in normal condition. Also cells in simulated microgravity were inclined to formed cell aggregated and accumulated structure.

5. Discussion

In this study, we sought to specify the influence of simulated microgravity condition on the differentiation potential of ADSCs to fibroblasts (18). The impact of this theorem that exposures to microgravity alter the gene expression of ECM (26-28) and the expression of ECM is one of the most important characters of fibroblast cells.

Fibroblasts represent a heterogeneous population of cells that play an important role in the maintenance of structural integrity within mesenchymal connective tissues. In recent years, they are widely used for engineering of tissues such as skin and blood vessels due to their role in wound healing and secretion of extra cellular matrix (ECM) components (4, 5, 29, 30). Because of ethical and safety problems in allogenic grafts and limitation in the acquirable amount of autogenic cells, nowadays stem cell differentiation has a much more attention for transplantation purpose (6). Some studies have demonstrated that MSCs from adult tissues are multipotent cells, capable to differentiate to fibroblasts (18, 31). These differentiated cells, derived biochemical manipulation of MSCs, have been shown to express some characteristics of fibroblast cells. However, efficiency of differentiated cells are varied and insufficient from one study to another which is most likely due to differences in inducing factors and the source of stem cells. Recently, much more attention has been paid to biophysical forces, including microgravity to manipulate stem cells (32-35). Microgravity, as a mechanical factor, has been confirmed to affect cell growth and gene expression. It can accelerate differentiation of MSCs and triggers cells to grow within three-dimensional aggregates that are an ideal condition for cell therapy and tissue engineering (23-25, 36).

Under microgravity condition, the cell morphology switches from a flattened spindle phenotype to an almost round phenotype that can cause by the destruction of microfilament structures. These changes may affect on inside signal cascade and the cell differentiation capability (37). In addition, microgravity cause changes in gene expression and metabolic activity in structures which are directly associated with mechanical forces such as ECM and adhesion molecules (37).

In this study, we showed that the CTGF-induced fibroblastic differentiation of ADSCs can be modulated by exposed to simulated microgravity. CTGF regulates differentiation, cell adhesion, gene expression, cell migration and proliferation. It exerts its function through binding to various cell surface receptors in a context-dependent manner, including integrin receptors (38-40) and cell surface heparan sulfate proteoglycans (HSPGs) (41).

The results of MTT assay suggest that CTGF affects cell proliferation. As seen in Figure 3B, cell proliferation increased after CTGF treatment. Our results showed that absorbance increased in both condition (1G environment and simulated microgravity condition) which represents the enhancement of cell proliferation and metabolic activity of cells. Previous studies have shown similar outcome on other cell types including fibroblast (42), periodontal ligament-derived cells (43), endothelial cells (44), and β-cell (45). Grotendorst and Duncan in 2005 demonstrated, the C-terminal domain of CTGF mediates cell proliferation. In this regard, our data indicated that simulated microgravity did not affect proliferation enhancement role of CTGF. According to MTT assay results, changes in the absorption were similar in both 1G environment and simulated microgravity condition after adding CTGF to culture medium and there were no statistically significant changes on it.

The commitment of ADSCs to the fibroblast cells is evidenced collectively by the reduction of mesenchymal markers like CD44 and simultaneous enhancement in the expression of fibroblast markers that most of them have important role in ECM formation (17). ECM synthesis is an important phase in wound healing. It helps to maintain the mechanical characteristics of the skin. Collagens, elastins and other proteoglycans are the major important substances of dermal ECM (46).

Quantitative PCR analyses confirmed that the expression of *CD44* down-regulated after CTGF treatment in normal condition (1G), which can due to the reduction of stem cell properties of ADSCs and differentiation to fibroblast. In contrast to normal condition, simulated microgravity increased the expression of CD44 (Fig. 4). These result confirmed data reported by Kumei *et al.* (26). They had shown that *CD44* expression was increased during the space flight that could be due to CD44 role in apoptosis or in cell adhesion.

Expression of *FN* and *FSP1* are increased after adding CTGF, but SMG didn't have statistically significant effect on expression of them. *FSP*1 is a specific fibroblast protein that indicates fibroblastic differentiation of ADSCs (47). According to the qPCR results, simulated microgravity may not impact on *FSP1* expression. Fibronectin is a glycoprotein of the ECM that binds to integrins. It is involved in numerous functions including cell adhesion, growth, migration, and differentiation. It is assembled into the ECM and supports the organs and tissues of an organism (48). Similar to *FSP1*, microgravity didn't have effect on fibronectin expression. Some other studies have suggested increasing of fibronection expression in microgravity condition (49-51). These reports stand in contrast to our study that may be due to differences in way of microgravity simulation, the source of cells and time of microgravity treatment.

Our data revealed that the mRNA level of collagens (type I and type III) increased after treatment by CTGF in 1G condition. Simulated microgravity condition caused further enhancing in expression of collagen type I and III. The mRNA level of *ELN* upregulated in both conditions too, but there was no statistically significant changes between 1G and simulated microgravity condition (Fig. 3). Collagen type I and III are two main collagenous fibers that contribute in tensile strength of connective tissue and ECM. Elastin is another fibrous protein that is critical for maintaining tissue elasticity (52). These proteins are also known as molecular markers which are related to fibroblast cells and fibroblastic differentiation (53, 54). With due attention to above information, it is suggested that simulated microgravity condition increases fibroblastic differentiation potential of MSCs *in vitro*.

Immunocytochemistry was done to stain the collagen type III and elastin expression at protein level on the 7th day of differentiation.

The Immunocytochemistry analysis confirmed the real-time PCR results, too. The expression of ColIII was obviously upregulated after CTGF-induced differentiation both in normal (1G) and simulated microgravity environment. In contrast to ColIII, the expression of ELN was slightly upregulated after differentiation of ADSCs to fibroblast only in simulated microgravity condition (Fig. 5F).

Meanwhile, previous studies have shown that microgravity induce cells to form aggregated structures and 3-D (tree-dimentional) spheroids (23-25). The same structures were observed in our study which is demonstrated in Figures 5C and 5F.

MMP1 breaks down the interstitial collagens, including type I and III and that is why the study of it along with ECM molecules would be important. In addition, it is one of the fibroblastic markers (18). Blaber *et al.* (2013) showed that during the space

flight (real microgravity environment), the expression of matrix metalloproteinases including MMP1 was obviously increased (47). Similar result was obtained in our study, too. We found that the expression of *MMP1* was upregulated after differentiation of ADSCs toward fibroblasts. Simulated microgravity situation caused further increasing in *MMP1* expression. These results emphasize that simulated microgravity may increases fibroblastic differentiation of MSCs.

In this study, we analyzed the expression of *FBN1* and *VIM*, too. Fibrillin-1 is a non collagenous polymeric glycoprotein that has a role in the formation of elastic fibre in the extra cellular matrix of connective tissues. Actually, fibrillin-1 stabilizes extra cellular matrix through formation of scaffold (55-57). We observed that the expression of *FBN1* was increased after differentiation of ADSCs by CTGF but simulated microgravity didn't have significantly effect on its expression. However, the expression of *FBN1* may suggest the influence of CTGF on induction of differentiation (18, 58). Expression changes of *VIM* were similar to *FBN1*. Vimentin is a type III intermediate filament (IF) protein that constitute the cytoskeleton along with tubulin microtubules and actin microfilaments. It is responsible for maintaining cell integrity (59). Previous studies have shown that components of cytoskeleton including microtubules are gravity sensitive and reorganized in microgravity that could be affect cell morphology and fate (60, 61). Based on the data obtained from our study, microgravity didn't have any effect on the expression of vimentin. Therefore, it seems that the effect of microgravity on cell morphology is applied through microtubules.

We have also evaluated the expression of *ITGB1*. We found that the expression of fibroblastic marker ITGB1 increased after fibroblastic differentiation (Fig. 4). Simulated microgravity condition caused further enhancing in expression of *ITGB1* (up to 5.5 fold) which have suggested the positive effect of microgravity on fibroblastic differentiation. Previous studies have reported conflicting results in this case that may be due to differences in way of microgravity simulation, the source of cells and time of microgravity treatment (26, 62, 63). Integrin is a membrane receptor that involved in cell-cell adhesion and cell-ECM interactions. It is also involved in recognition in a variety of processes including tissue repair and wound healing (64).

6. Conclusion

In conclusion, for the first time, we differentiated ADSCs to fibroblast cells under simulated microgravity condition. Our findings provide a new strategy for differentiation of ADSCs to fibroblastic cells and probably other cell types. According to gene and protein expression results, specially the expression of collagen type I and III, *MMP1*, *ITGB1* and *FSP1*, the simulated microgravity could enhance the differentiation of MSCs towards fibroblast. Based on our results, microgravity could be used as a new way besides old methods to increase the efficiency of cell transplantation. Meanwhile, microgravity simulation had no adversely affects on viability of the cells and could be used as new environment to successfully manipulate cells. Simulated microgravity condition or other forces (shear stress) may be able to differentiate fibroblasts without any other chemical or biological reagents.

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